

Acetylcholine exerts additive and permissive but not synergistic effects with insulin on glycogen synthesis in hepatocytes

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Abstract Parasympathetic (cholinergic) innervation is implicated in the stimulation of hepatic glucose uptake by portal vein hyperglycaemia. We determined the direct effects of acetylcholine on hepatocytes. Acute exposure to acetylcholine mimicked insulin action on inactivation of phosphorylase, stimulation of glycogen synthesis and suppression of phosphoenolpyruvate carboxykinase mRNA levels but with lower efficacy and without synergy. Pre-exposure to acetylcholine had a permissive effect on insulin action similar to glucocorticoids and associated with increased glucokinase activity. It is concluded that acetylcholine has a permissive effect on insulin action but cannot fully account for the rapid stimulation of glucose uptake by the portal signal. © 2007 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

The extent by which the liver clears glucose from the blood in conditions of elevated blood glucose concentration is dependent on the glucose load, the route of delivery of glucose to the liver and the insulin to glucagon ratio [1]. Insulin is more effective at stimulating hepatic glucose uptake and glycogen storage when glucose is delivered via the portal vein as opposed to the hepatic artery or the peripheral circulation [2–4]. This mechanism which is known as the “Portal Signal” is dependent on an intact nerve supply to the liver [5–7] and is thought to involve glucose sensing in the portal vein [8] and altered transmission of parasympathetic (cholinergic) and sympathetic (noradrenergic) stimuli to the liver [9–11]. Activation of sympathetic nerves directly stimulates glucose output by hepatocytes through the neurotransmitters noradrenaline and ATP via adrenergic and P2Y receptors, respectively [12]. It is generally assumed that activation of parasympathetic nerves stimulates hepatic glucose uptake and suppresses glucose production through release of acetylcholine. This is based largely on studies in vivo and on

the in situ perfused liver. Pharmacological inhibition of cholinergic nerves with atropine counteracted insulin-stimulated hepatic glucose uptake in the cat, dog and rat [4,9,13–15]. However, studies on freshly isolated hepatocytes have not provided evidence for greater efficacy of acetylcholine compared with insulin [16]. Hepatocytes in monolayer culture are a better model for studying insulin action, because pre-culture with low concentrations of glucocorticoid has a permissive effect on insulin action [17,18]. In this study, we used hepatocytes in monolayer culture to evaluate the direct effects of acetylcholine on glucose metabolism and insulin action during either acute exposure to acetylcholine or after pre-exposure to acetylcholine.

2. Materials and methods

2.1. Isolation and culture of hepatocytes

Hepatocytes were isolated by collagenase perfusion of the liver from male Wistar rats fed ad libitum [19]. The cells were seeded in minimum essential medium (MEM) supplemented with 5% newborn calf-serum and after cell attachment (4 h) they were cultured in serum-free MEM containing 5 mM glucose and the concentrations of dexamethasone or acetylcholine as indicated [19].

2.2. Metabolic incubations

After 16 h pre-culture in serum-free MEM without or with dexamethasone or acetylcholine, the medium was replaced by serum-free MEM containing 15 mM glucose and other additions as indicated and incubations were for 1–3 h. For determination of glycogen synthesis the medium was supplemented with [U - ^{14}C] glucose (2 μ Ci/ml). Glycogen synthesis was determined after 3 h incubation by ethanol precipitation as previously described [19]. Parallel 1 h incubations without radiolabel were performed for determination of glucose 6-phosphate (glucose 6-P) [20] and enzyme activities.

2.3. Enzyme and protein determination

For determination of glucokinase, cells were permeabilised with digitonin and glucokinase was assayed as in [21]. Phosphorylase-a and glycogen synthase were assayed as in [19]. cAMP phosphodiesterases (PDE), type-3 (PDE3) and type-4 (PDE4) were assayed as in [22] except that alkaline phosphatase (0.3 U/sample) was used instead of snake venom. Phosphorylation of protein kinase B on ser-473 was determined by immunoblotting [23].

2.4. mRNA determination

Total RNA (1 μ g) was extracted from hepatocytes using Trizol (Invitrogen) and treated with RNase free DNase I (Roche). cDNA (50 ng) was synthesised using Superscript II (Invitrogen) in the presence of random hexamers (GE Healthcare UK Ltd.) and dNTP mix (Bioline). Real-time RT-PCR analysis was performed starting with 50 ng cDNA in a PCR reaction (10 μ l) containing 0.5 μ M primers, 3 mM $MgCl_2$ and 1 \times Light Cycler FastStart DNA Master SYBR Green I using a Light Cycler (Roche).

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Abbreviations: GK, glucokinase; glucose 6-P, glucose 6-phosphate; MEM, minimum essential medium; OBAA, 3-(4-octadecyl)-benzoyl-acrylic acid; PDE, cAMP phosphodiesterase; PEPCK, phosphoenolpyruvate carboxykinase

The primers used were; glucokinase (GK), sense, 5'-CAACATCGTAGGACTTCTCCG-3', anti-sense, 5'-GGCGGTCTTCATAGTAGCAG-3'; phosphoenolpyruvate carboxykinase (PEPCK), sense, 5'-TGGCTACGTCCCTAAGGAA-3', anti-sense, 5'-GGTCCTCCAGATACTTGTCGA-3'. PCR was performed by pre-incubation for 10 min at 95 °C for initial denaturation followed by 40 cycles consisting of 95 °C for 15 s, 58 °C for 7 s and 72 °C for 15 s were performed. Cyclophilin was measured as an invariant control and the relative changes in mRNA for a given gene were corrected for cyclophilin mRNA values. PCR specificity and product length were checked by gel electrophoresis.

2.5. Analysis

Results are expressed as means \pm S.E.M for the number of hepatocyte preparations indicated. Statistical analysis was by the paired *t*-test, except where indicated which was by 2-way ANOVA.

3. Results

3.1. Acute effects of acetylcholine on glycogen metabolism

Because glucocorticoids have a permissive effect on insulin action [17] we compared the effects of acetylcholine and a maximally effective concentration of insulin (10 nM) in hepatocytes pre-cultured without or with low concentrations of dexamethasone (Fig. 1). Acetylcholine caused a small stimulation of glycogen synthesis after pre-culture without dexamethasone that was comparable to the stimulation by insulin (Fig. 1A and B). However, unlike insulin action, the stimulation by acetyl-

choline was not enhanced by pre-culture with dexamethasone. The effects of acetylcholine and insulin were additive and there was no evidence for synergy. Like insulin action, the stimulation of glycogen synthesis by acetylcholine was associated with inactivation of phosphorylase (conversion of phosphorylase-a to phosphorylase-b) and there was an inverse correlation between glycogen synthesis and phosphorylase-a during incubation with insulin and/or acetylcholine (Fig. 1C and D). Acetylcholine and insulin caused comparable activation of glycogen synthase and the combined effects were less than additive (insulin, 25.7 ± 2.5 , acetylcholine 19.3 ± 8.9 ; acetylcholine + insulin 32.4 ± 5.0 , % increase above control). The stimulation of glycogen synthesis by acetylcholine was blocked by 10 μ M atropine (control 13.2 ± 4.3 ; acetylcholine 16.1 ± 4.5 , $P < 0.005$; atropine 13.9 ± 3.9 ; atropine + acetylcholine, 14.2 ± 3.0 , $P > 0.05$, nmol/3 h/mg⁻¹) consistent with involvement of muscarinic receptors [24]. Unlike insulin action acetylcholine did not cause phosphorylation of protein kinase B on ser-473 (results not shown) and stimulation of glycogen synthesis was blocked by 3-(4-octadecyl)-benzoylacrylic acid (OBAA) (Fig. 2) an inhibitor of phospholipase A2 [25].

3.2. Counterregulatory effects of acetylcholine on glucagon action

Glucagon stimulates glycogenolysis acutely by activation of phosphorylase (conversion of phosphorylase-b to phosphory-

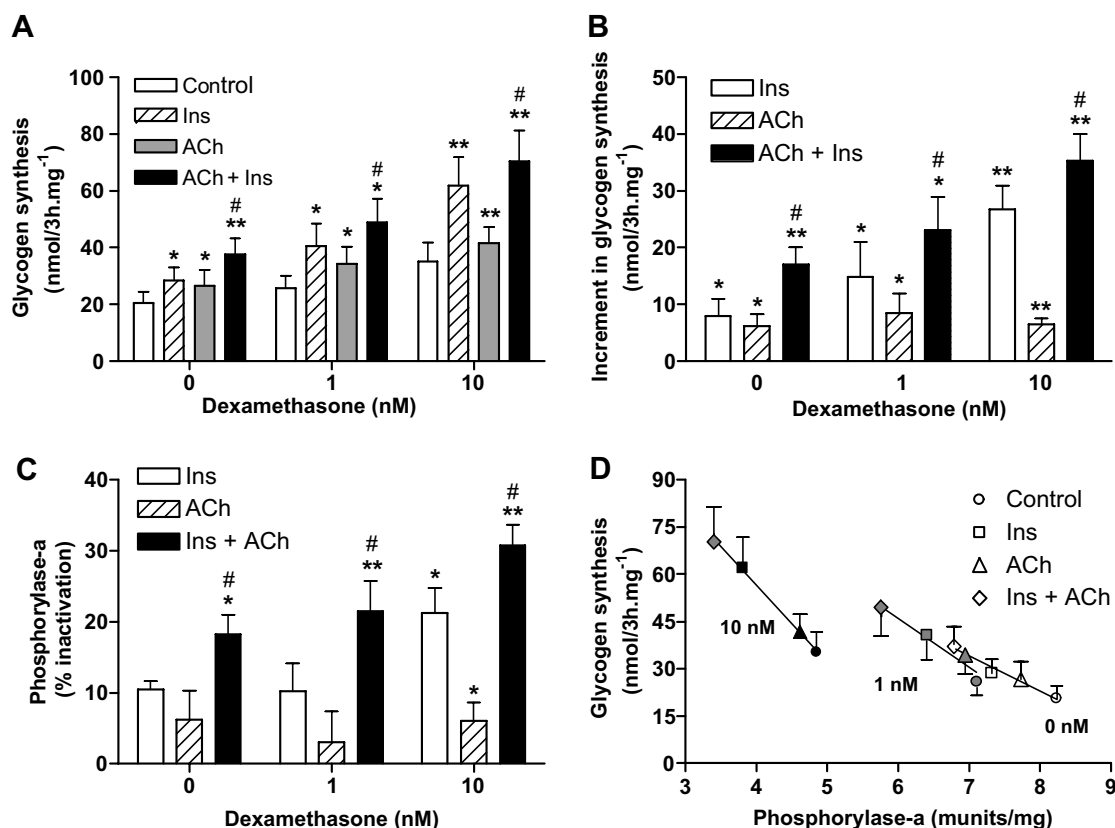


Fig. 1. Short-term effects of acetylcholine and insulin on glycogen synthesis and phosphorylase activity. Hepatocytes were pre-cultured without or with dexamethasone (1 or 10 nM) and then incubated without or with 250 μ M acetylcholine (ACh) and 10 nM insulin (Ins) for determination of glycogen synthesis and phosphorylase-a activity as described in Section 2. (A) Rates of glycogen synthesis. (B) Increment in glycogen synthesis caused by acetylcholine and insulin. (C) Inactivation of phosphorylase (% decrease relative to control). A–C. Means \pm S.E.M., $n = 5$ (A and B) or 4 (C). * $P < 0.05$, ** $P < 0.005$ relative to control; # $P < 0.05$ relative to insulin. (D) Correlation between glycogen synthesis (nmol/3 h/mg⁻¹) and phosphorylase-a (mU/mg), means of $n = 5$, in the absence of dexamethasone (0 nM) or in the presence of 1 nM or 10 nM dexamethasone as indicated.

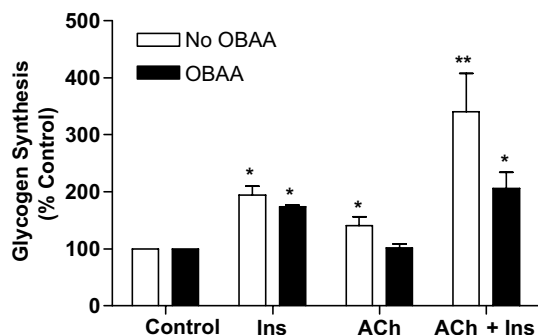


Fig. 2. Counteraction of acetylcholine effect by OBAA. Hepatocytes were pre-incubated without or with 10 μ M OBAA for 15 min before incubating without or with 250 μ M acetylcholine (ACh) and 10 nM insulin (Ins) for determination of glycogen synthesis (% control). Means \pm S.E.M., $n = 5$. * $P < 0.05$, ** $P < 0.005$ relative to corresponding control.

lase-a) and it stimulates gluconeogenesis chronically by induction of gluconeogenic enzymes. Acetylcholine like insulin partially counteracted the effects of glucagon on activation of phosphorylase and the effects of insulin and acetylcholine were additive at 0.5 nM glucagon but not at higher concentrations

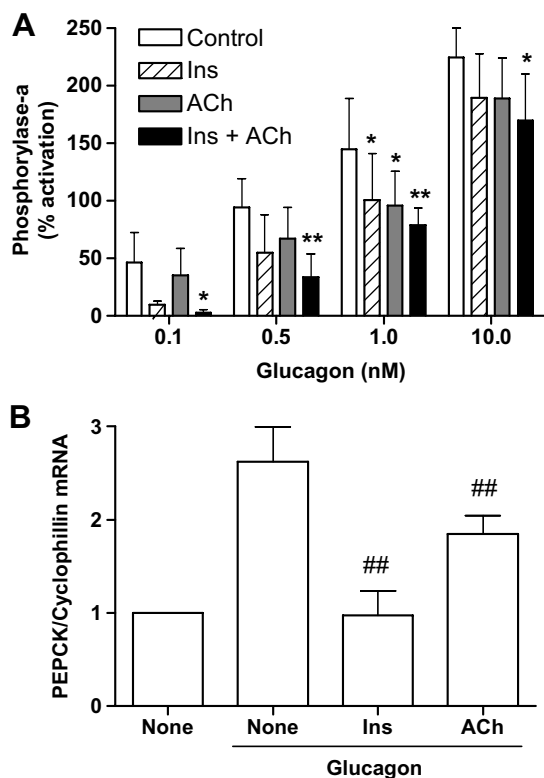


Fig. 3. Acetylcholine partially counteracts glucagon action. (A) Hepatocytes were pre-incubated without or with 250 μ M acetylcholine (ACh) and/or 10 nM insulin (Ins) for 60 min before addition of glucagon (5 min) at concentrations shown for determination of phosphorylase-a. Means \pm S.E.M., $n = 4$, * $P < 0.05$, ** $P < 0.005$ relative to control, 2-way ANOVA. (B) Hepatocytes were pre-cultured with 100 nM dexamethasone and then incubated without or with 250 μ M acetylcholine for 10 min before addition of 0.2 nM glucagon (3 h) for determination of PEPCK mRNA as described in Section 2. Means \pm S.E.M., $n = 6$, ## $P < 0.005$ relative to glucagon alone.

(Fig. 3A). Acetylcholine suppressed PEPCK mRNA levels but with lower efficacy than insulin (Fig. 3B).

3.3. Permissive effect of acetylcholine on insulin action

Because hepatic denervation is associated with insulin resistance and decreased hepatic glycogen storage [14,26] implicating a chronic role for parasympathetic stimulation in regulating hepatic glucose metabolism we tested the effects of pre-culture with acetylcholine on the subsequent short-term response to insulin. Pre-culture of hepatocytes with acetylcholine mimicked the permissive effect of dexamethasone on insulin action on glycogen synthesis and inactivation of phosphorylase (Fig. 4A–D). These effects of acetylcholine were associated with an increase in glucokinase activity (Fig. 4E) and in the glucose-dependent increase in glucose 6-P (Fig. 4F), indicating that the inactivation of phosphorylase and stimulation of glycogen synthesis can be in part explained by the elevated glucose 6-P, which causes depletion of phosphorylase-a [27]. Acetylcholine potentiated the increase in glucokinase mRNA caused by insulin (Fig. 5) but had no effect in the absence of insulin (results not shown). The effects of acetylcholine are unlikely to be due to changes in cAMP, because acetylcholine unlike dexamethasone [28] did not affect the activity of PDE3 and PDE4 (Fig. 6).

4. Discussion

The contribution of the Portal Signal to regulation of hepatic glucose balance has been determined experimentally by comparing rates of hepatic glucose production or uptake when a glucose load is administered either through the portal vein or the hepatic artery/peripheral circulation at the same clamped level of insulin. These studies on both man and animal models support a major contribution of the Portal Signal to stimulation of hepatic glucose uptake in hyperinsulineamic conditions [1–3]. The involvement of the parasympathetic innervation in the Portal Signal is supported by the inhibitory effect of atropine, an antagonist of muscarinic receptors [4,9] and by chronic denervation [5–7] though not by acute cooling of the vagal nerve [29,30]. If the contribution of cholinergic mechanisms to the stimulation of hepatic glucose uptake were due to a direct effect of acetylcholine on hepatocytes as opposed to either haemodynamic changes or indirect effects involving release of other neurotransmitters (or incretins), then acetylcholine would be expected to have either a large stimulatory effect on glycogen synthesis in comparison with insulin action or alternatively a synergistic effect with insulin. An indication of synergy between acetylcholine and insulin by either direct or indirect mechanisms was provided in the in situ perfused rat liver [4]. However, this experimental model cannot exclude indirect effects on the liver.

4.1. Lack of synergy during acute exposure to insulin and acetylcholine

In this study, we used short-term primary hepatocyte cultures (within 24 h of isolation) to compare the effects of acetylcholine and insulin on glucose metabolism. The key advantage of this model is that it allows pre-incubation of hepatocytes in defined substrate and hormone conditions prior to study of the short-term effects of insulin. In this model, low concentrations

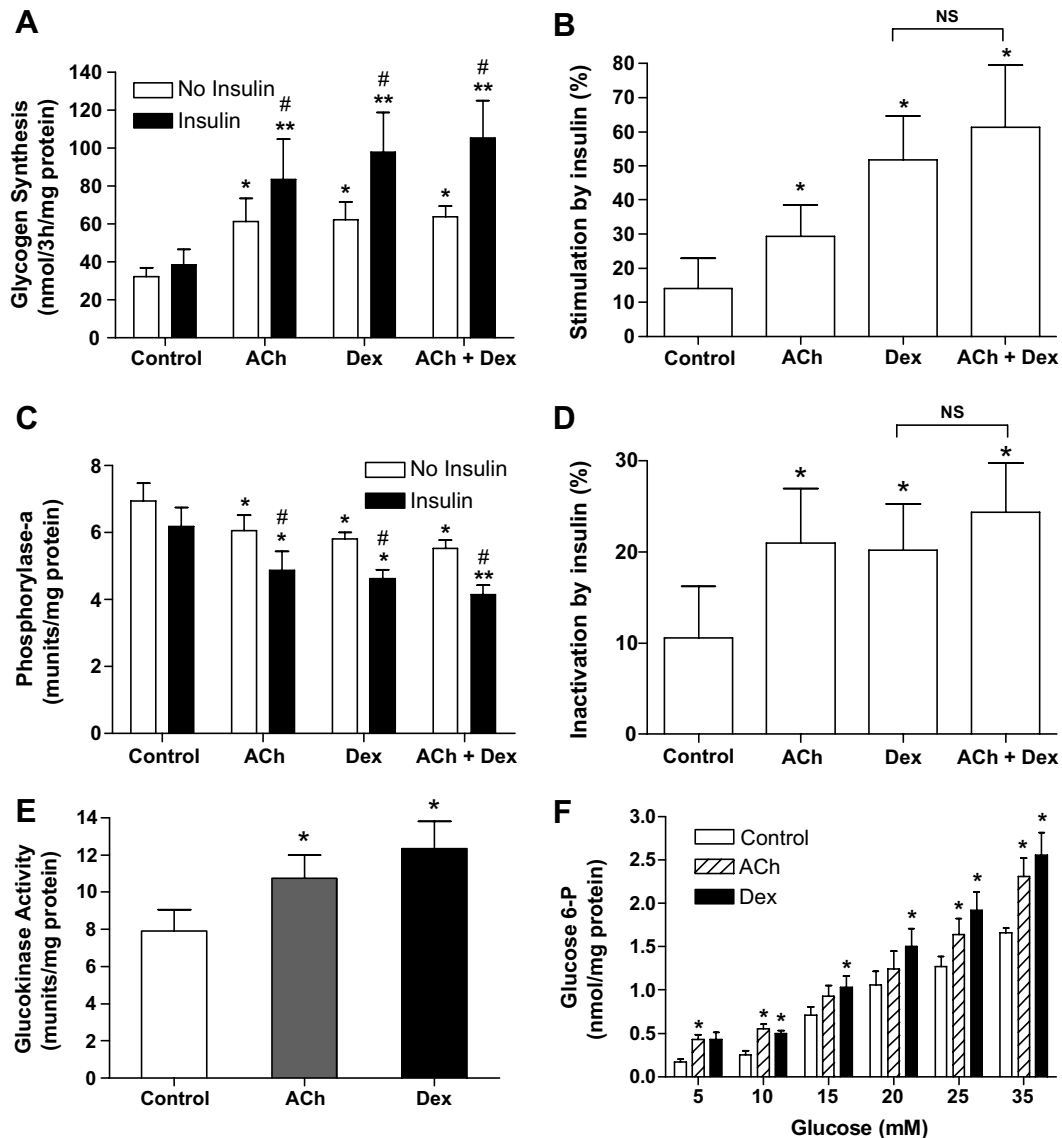


Fig. 4. Pre-exposure to acetylcholine mimics the permissive effect of glucocorticoid. Hepatocytes were pre-cultured for 16 h without or with 50 μ M acetylcholine (ACh) and 10 nM dexamethasone (Dex). The effects of insulin on glycogen synthesis (A and B) and phosphorylase-a (C and D) activity were then determined. (E) Glucokinase activity. (F) Glucose 6-P determined after 1 h incubation with the glucose concentrations shown. * $P < 0.05$, ** $P < 0.005$ relative to corresponding control; # $P < 0.05$ relative to no insulin.

of glucocorticoid have a permissive effect on insulin stimulation of glycogen synthesis which is mediated by inactivation of phosphorylase [17]. The experiments testing the effects of acute exposure to acetylcholine show that the stimulation of glycogen synthesis is either smaller (<50%) than the stimulation by insulin in cells pre-treated with glucocorticoid or it is at best comparable to the small effect of insulin in cells pre-cultured without glucocorticoid. There was no evidence for synergy between acetylcholine and insulin on either glycogen synthesis or phosphorylase inactivation. The counteraction of glucagon induction of PEPCK mRNA levels was also less than the effect of insulin.

Intraportal hyperglycaemia accounts for at least 60% of the stimulation of hepatic glucose uptake, and is more rapid than insulin-stimulated hepatic glucose uptake [36,37]. The lower efficacy of acetylcholine compared with insulin and the lack of synergy does not support a major direct role for acetylcho-

line in mediating the stimulation of hepatic glucose uptake by intraportal hyperglycaemia.

The counteraction by atropine of the glycogenic effect of acetylcholine is consistent with involvement of muscarinic receptors [24]. The M1–M5 muscarinic receptors are coupled to either Gq proteins resulting in activation of phospholipase C and phospholipase D leading to activation of phospholipase A2 or to Gi proteins leading to a decrease in cAMP [31]. The counteraction of the action of acetylcholine by the phospholipase A2 inhibitor implicates a role for bioactive mediators downstream of phospholipase A2 in stimulating glycogenesis. Although activation of phospholipase A2 can mediate the cytotoxic effects of arachidonic acid in hepatocytes [32], it is possible that modest activation of the enzyme can mimic insulin action by an analogous mechanism as has been shown for reactive oxygen species (ROS), which are implicated in insulin action [33]. ROS generated endogenously and also exogenous

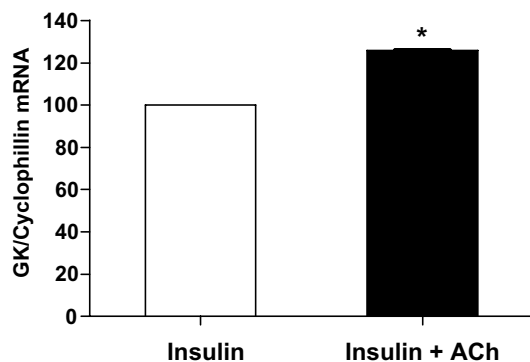


Fig. 5. Acetylcholine potentiates insulin induction of glucokinase mRNA. Hepatocytes were cultured for 16 h with 10 nM insulin without or with 200 μ M acetylcholine. Means \pm S.E.M., $n = 5$, * $P < 0.005$.

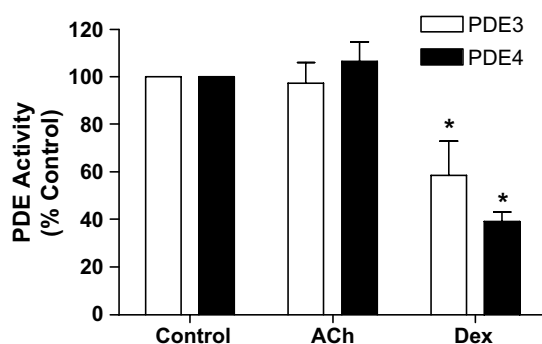


Fig. 6. Acetylcholine unlike dexamethasone does not affect PDE3 and PDE4 activity. Hepatocytes were pre-cultured for 16 h without of with 10 nM dexamethasone (Dex) or 200 μ M acetylcholine (ACh) as indicated. Means \pm S.E.M., $n = 3$, * $P < 0.05$ relative to control.

hydrogen peroxide mimic insulin action by lowering PEPCK mRNA levels in hepatoma cells [34] and by stimulating glucose metabolism in adipocytes [33] and glycogen synthesis in hepatocytes (A. Zaidi and L. Agius, unpublished results). PI-3-kinase independent and Gi-alpha dependent pathways have been implicated in the mechanism by which insulin generates ROS [33,35]. The additive as opposed to synergistic effects of insulin and acetylcholine on glycogen synthesis are consistent with generation of ROS via Gi-alpha (insulin [35]) and Gq (acetylcholine), which may impact glucose metabolism through a common mechanism.

4.2. Permissive effect of acetylcholine on insulin action

Pre-culture of hepatocytes with low concentrations of glucocorticoids has a permissive effect on insulin action on glycogen synthesis, inactivation of phosphorylase [17] and induction of glucokinase mRNA [18]. This effect is not due to increased insulin binding, activation of the insulin receptor kinase or activation of protein kinase B which are not affected by glucocorticoids [17]. The additive effects of insulin and acetylcholine in the acute exposure studies and the lack of effect of acetylcholine on phosphorylation of protein kinase B, is consistent with a signaling pathway independent of protein kinase B. One of the well-characterized effects of glucocorticoids in hepatocytes is the suppression of cAMP-phosphodiesterase

activity [28]. However, acetylcholine unlike dexamethasone did not affect the activities of PDE3 and PDE4. It seems unlikely therefore that altered cAMP turnover accounts for the permissive effects of acetylcholine. The lack of additive effects of acetylcholine and glucocorticoids in these experiments suggests that whilst glucocorticoids may activate various signaling pathways, a component shared with the acetylcholine mechanism may mediate the permissive effect on insulin action. The permissive effect of acetylcholine on insulin action is consistent with previous *in vivo* studies showing that chronic liver denervation results in a reduction in insulin responsiveness that is reversed by administration of acetylcholine [26].

In summary, this study supports the conclusion that whilst both the acute and chronic effects of acetylcholine on glycogen synthesis are consistent with an anabolic role on glycogen metabolism that is mediated by either inactivation of phosphorylase or by increased glucokinase activity, the magnitude of these direct effects of acetylcholine is not sufficient to explain the large stimulation of hepatic glucose uptake by intraportal infusion of glucose [1,2] described as the "Portal Signal". The possibility needs to be considered that other neurotransmitters (or incretins) with greater efficacy than acetylcholine and acting independently or synergistically with insulin and acetylcholine may be involved in the stimulation of hepatic glucose uptake by intraportal hyperglycaemia.

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